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Plasma-chemical modifications of cellulose for biomedical applications

Abstract: A 6-carboxycellulose (in medicine known as "oxidized cellulose" or "oxycellulose") is one of the cellulose derivatives popular in the field of surgery. Health products based on oxidized cellulose are great local hemostatics with unique bactericidal and fully bioabsorbable effects. Traditional process of native cellulose oxidation is described as a complex radical reaction in strong acidic liquid medium doped by toxic nitrous radicals (NO*). Our plasma-chemical reaction demonstrates a new synthesis method of oxidized cellulose with unique bactericidal effect. This plasma-chemical treatment is based on atmospheric plasma discharge in liquid medium leading to the oxidation of polysaccharide molecules resulting in oxycellulose. Final oxycellulose properties were evaluated by infrared spectroscopy and carboxyl content determination. The biological impact showed a strong germicidal effect.

Keywords: oxidized cellulose, oxycellulose, antibacterial, plasma-chemical treatment, plasma

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1 Introduction

The term oxidized cellulose refers to any material produced by the oxidation of primary alcohol groups on the cellulose chain. During the process of oxidation, aldehyde, ketone and carboxyl groups can be formed, depending on the nature of the oxidizer and the parameters of the process. The application of modified cellulose as a dressing for chronic and burn wounds has been described several times as beneficial for wound healing. Oxidized cellulose is an important substance for many medical applications due to its local hemostatic and bactericidal effect against a wide range of pathogenic microorganisms and its healing effects. There is a generally accepted notion that these unique effects cause a presence of carboxylic groups in its chemical structure [1-3]. The major problem is the difficulty of producing materials, which are homogenous in chemical and physical properties. Several of the oxidants employed are environmentally toxic as they contain or produce nitrous radicals NOx [4-6]. The main aim of this work was to develop a new cellulose oxidation process with benefit of plasma discharge in the liquid medium.

It is well known that plasma discharge treatment (including irradiation by UV light, X-ray and substrate bombardment with electrons, ions and radicals) leads to the degradation of polymeric chains, chemical bond cleavage and creation of free radicals [7]. Subsequent chemical reactions of transient, highly reactive species can result in forming oxidized structures with biological impact. In order to verify and compare biological activity of pure microcrystalline cellulose (MC) and plasma-chemical modified microcrystalline cellulose (MMC) the antibacterial properties were evaluated. Bacterial presence complicates wound healing. Therefore, using surgical dressing stabilizing or reducing bacterial amount is therefore highly important.

We used real-time antibacterial measurement of MMC based on *Escherichia coli*, where its viability is positively correlated with bioluminescence as an easy detectable marker [8]. *E. coli* K-12 was transformed with plasmid including a modified bacterial luciferase gene (luxABCDE) originating from the chromosomal (luxCDABE) gene of the Gram-negative soil bacterium *Photorhabdus luminescens*. This method is frequently used for determination of

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antibacterial activity in biological samples such as complement or antibacterial peptides [9].

In this study, we used the RF (13.56 MHz) plasma jet operated at atmospheric pressure. Application of admixture gas such as oxygen or carbon dioxide in working gas (argon) is beneficial because it leads to direct formation of reactive oxidative species. However, although increasing the volume of supplement gases in the plasma source can lead to a higher amount of oxidative particles, their overall effect causes various instabilities in discharge. In our experiment we set the maximum volume of admixtures without observable instabilities of the plasma jet.

2 Experimental procedure

2.1 Materials

Microcrystalline cellulose (MC) suitable for medicinal applications, was used as starting cellulose source, was obtained from Penta (Czech Republic). Hydrogen peroxide (30%, p.a. grade) was purchased from MACH CHEMIKALIE (Czech Republic). Argon (purity 99.996%), oxygen (purity 99.5%) and carbon dioxide (purity 99.5%) were purchased by Linde Gas (Czech Republic).

2.2 Plasma-chemical reaction

The plasma source was carried out by jet of atmospheric pressure radiofrequency hollow cathode – so called plasma pencil [10,11]. This discharge has a single-electrode configuration and is operating in argon working gas. Fig. 1 shows the experimental set up of the plasma-chemical reactor. The radiofrequency generator (13.56 MHz, Dressler Cesar) operates with 180 W of the mean power. The matching was automatically adjusted to maintain reflected power below 1 W. The plasma-chemical reactor design consists of a hollow cathode with silica discharge capillary in a Y shape and a 100 mL reagent glass bottle. Power from the generator and input for argon were connected to one branch of Y capillary. The admixture of oxygen and/or carbon dioxide could be added to the second Y branch and flow rate was controlled by a flow controller. In our experiment we set the maximum volume of admixtures without visible instabilities of plasma jet. This flow rate was therefore different for each kind of admixture and is presented in Table 1. Through the discharge tube the flow of argon (5.0 slm (standard litre per minute)) was maintained by a second flow-controller.

Figure1: Photo of plasma-chemical treatment MC and scheme of the experimental plasma-chemical reactor.

The bottom of the reagent bottle is grounded by metal belt from the outer side. During plasma-chemical treatment the end of discharge capillary was submerged under the level of liquid in the reagent glass bottle.

2.3 Preparation of samples

5.0 g of microcrystalline cellulose was added to 50 mL of distilled water or hydrogen peroxide solution. Concentrated hydrogen peroxide (30%) was diluted with distilled water to obtain 5, 10 and 15% (v/v) H_2O_2. The MC suspension, with either H_2O or H_2O_2 as a liquid medium, was placed

into the reagent bottle and argon plasma discharge was initiated by Tesla coil. In some cases, admixture gas of O_2 or CO_2 was used in order to increase the plasma treatment efficiency. After plasma-chemical reactions (taking for 5 or 15 minutes) the samples were filtered off. Separated cellulose material was air-dried at room temperature (24°C) to a constant weight. Parameters for plasma-chemical treatment are summarized in Table 1.

Table 1: Parameters for MC plasma-chemical treatment.

liquid medium	sample	treatment time [min]	plasma gas
Water	A1	5	Ar (5 slm)
	A2	15	Ar (5 slm)
	A4	15	Ar (5 slm) + O_2 (9 mL min^{-1})
	A6	15	Ar (5 slm) + CO_2 (15 mL min^{-1})
5% H_2O_2	B1	5	Ar (5 slm)
	B2	15	Ar (5 slm)
	B4	15	Ar (5 slm) + O_2 (9 mL min^{-1})
	B6	15	Ar (5 slm) + CO_2 (15 mL min^{-1})
10% H_2O_2	C1	5	Ar (5 slm)
	C2	15	Ar (5 slm)
15% H_2O_2	D1	5	Ar (5 slm)
	D2	15	Ar (5 slm)

2.4 Infrared analysis

Attenuated total reflectance Fourier transform infra-red spectra (ATR-FTIR) were measured by Bruker Vertex80V spectrometer. Diamond was used as the ATR crystal material. Spectral range was set from 4000 to 600 cm^{-1} with resolution of 4 cm^{-1}. Each spectrum consists of 100 scans. The spectra were evaluated by OPUS software (version 6.5) using "rubber-band" baseline correction. For comparison purposes FTIR spectra were normalized.

2.5 Determination of carboxyl content

The carboxyl content of plasma-chemical treated samples was determined by titration according to United States Pharmacopoeia [12]. Briefly, about 0.5 g of the sample was accurately weighed and dissolved in 5 mL of 0.5 M solution of sodium hydroxide (NaOH). Five minutes later 50 mL of distilled water was added and the solution was titrated with standardized 0.1 M hydrochloric acid (HCl) using phenolphthalein as an indicator. The volume of HCl solution was corrected for the blank. The carboxylic content in the sample was calculated from the following relationship:

$$COOHcontent(\%) = \frac{100 * (V_0 - V) * f * c_{HCl} * M_{WCOOH}}{m}$$

Where V_0 is the volume of HCl consumed in titration for blank in L, V is volume of HCl consumed for the sample in L, f is concentration factor, c_{HCl} is molar concentration of HCl solution and m is weight of the sample in g. $M_{W\,COOH}$ is molecular weight of COOH groups (Mw = 45 g mol^{-1}).

2.6 Antibacterial properties of plasma-chemically modified cellulose

The biological effect of the samples was examined *via* bioluminescent (BL) bacteria. Genetically modified *E. coli* K-12 capable of bioluminescence were exposed to cellulose derivatives which led to diminishment of bacterial viability visualised by real time BL measurement. Concentrated stock bacterial suspension was prepared according to Atosuo *et. al.* [8] and stored at temperature −80°C. Final concentration of bacterial cells in applied suspension was set to approximately 380 000 cells per 100 µL. This suspension (200 µL) was mixed with 5, 10 or 15 mg of plasma-chemically modified samples partly dissolved in phosphate buffer (100 µL) in wells of a white flat-bottom 96-well microtiter plates (Thermo Scientific, Czech Republic). MC cellulose samples (5, 10 or 15 mg) were used as an appropriate control. Light (490 nm) generated by enzymatic reaction in bioluminescent bacteria was measured continuously during 60 minutes by luminometer LM-01T (Immunotech, Czech Republic) at laboratory temperature. Results are expressed in relative light units (RLU). Average integral under the kinetic curve was compared with control and the percentage of bacterial killing was calculated. Experiments were repeated independently three times.

3 Results and discussion

3.1 ATR-FTIR spectroscopy

The selected parts of ATR-FTIR spectra of plasma-chemically treated MC samples are shown in Figs. 2 and 3. The influence of the time of plasma treatment and

liquid medium in plasma source of groups A, B, and D on the MC plasma-chemical treatment is shown in Fig. 2. In comparison with non-treated microcrystalline cellulose the FTIR spectra of all treated materials appeared to be almost similar besides the new characteristic bands at region 1695 - 1775 cm^{-1} and increasing band between 1550 and 1695 cm^{-1}. Compared to the Wei *et al.* [13] conclusions, new bands proved the presence of carboxyl groups at 1740 cm^{-1} in the obtained products. Absorbance at about 1640 cm^{-1} is related to the bending mode of H_2O molecules [13]. After the plasma treatment in distilled water (sample A1 and A2) the increasing intensity of 1640 cm^{-1} band was observed while typical absorbance of carboxyl groups at 1740 cm^{-1} was not detectable. However, experiments conducted in an environment of hydrogen peroxide always led to formation of new carboxylic group absorbance bands. The bending mode of H_2O molecules increases with higher amount of H_2O_2 corresponding to higher polymer hydrophilicity due to the carboxyl groups. Moreover, the longer the plasma treatment in 15% H_2O_2 suspension (sample D1 and D2) the higher amount of hydroxyl and carboxyl groups of modified MC are detected by ATR-FTIR. Novel oxycellulose material is thus produced by gradual non-specific oxidation process of MC due the plasma-chemical treatment.

Fig. 3 shows the effect of plasma admixtures composition in samples of the group B (5% H_2O_2) on the chemical structure. Intensity of absorbance of both carboxyl groups at 1740 cm^{-1} and the bending of H_2O molecules at 1640 cm^{-1} decreased in the order of Ar+CO_2 > Ar+O_2 > Ar > MC (original non plasma treated sample).

Data from ATR-FTIR measurements were divided into two regions. Characteristic bands in the range 1550 – 1695 cm^{-1} (bending mode of H_2O molecules) and 1695 – 1775 cm^{-1} (-COOH stretch) were subsequently integrated. The obtained integrals are shown in Table 2. These data were compared with those from a titration method. Larger area corresponds to a higher degree of sample oxidation. Samples treated in water (A1 - A6) were not significantly chemically changed while samples treated in H_2O_2 (groups B – D) led to an increased amount of oxidation groups. Samples in group D (15% H_2O_2) resulted in the maximum increase of integrated area (up to 500 %) in comparison with untreated samples. The longer treatment time and the higher the hydrogen peroxide concentration the more effective the oxidation process was observed.

3.2 Determination of carboxylic group content

The amount of –COOH group (%) and integrated area from ATR bands are compared in Table 2. Carboxylic

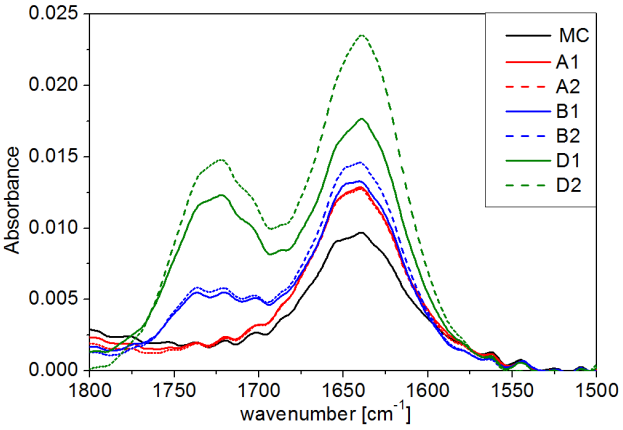

Figure 2: The ATR-FTIR spectra show the influence of H_2O_2 concentration (A - water, B - 5%, and D - 15%) and the time of plasma treatment in samples of the group (straight curve for 5 min and dashed curve for 10 min of plasma treatment). MC curve belongs to non-treated microcrystalline cellulose

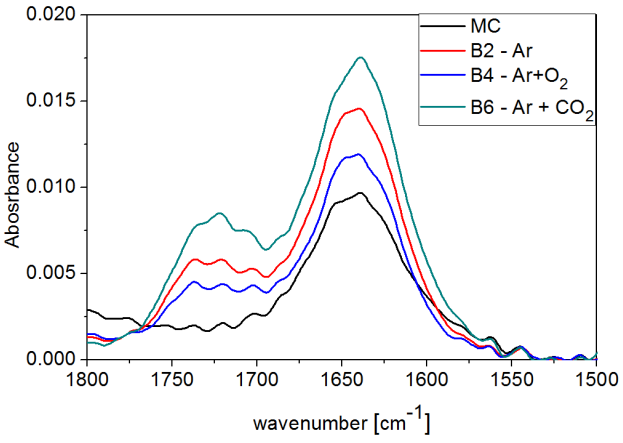

Figure 3: The ATR-FTIR spectra show the influence of admixture in samples of the group B (5% H_2O_2). Red curve presents a 15 min plasma treatment with pure argon, blue the admixture of oxygen and teal admixture of carbon dioxide. MC belongs to non-treated microcrystalline cellulose.

group content of samples obtained from the titration method correspond well with the ATR integrated data of carboxylic bands. The peak area of the MC band between 1695 – 1775 cm^{-1} (–COOH groups) did not change after the plasma treating in water (group A). However, while the plasma treating proceeded in H_2O_2 solution the amount of -COOH groups increased with the H_2O_2 concentration. The maximum of about 5% of -COOH groups were obtained by treating the MC sample by plasma in 15% (v/v) H_2O_2.

Table 2: Integrated areas from ATR spectroscopy and determination of carboxylic content.

Liquid medium	sample	Plasma discharge	Treatment time [min]	ATR - Integrated Area [%]		ATR Ratio of integrated area COOH:H_2O	% COOH from titration
				H_2O 1550 -1695 cm^{-1}	COOH 1695 -1775 cm^{-1}		
-	MC	-	-	100%	100%	23.4%	0
Water	A1	Ar	5	127%	101%	18.6%	0
	A2	Ar	15	114%	94%	19.3%	0
	A4	Ar + O_2	15	123%	116%	22.1%	0.2
	A6	Ar + CO_2	15	130%	128%	23.0%	0.1
5% H_2O_2	B1	Ar	5	129%	209%	38.0%	1.9
	B2	Ar	15	139%	217%	36.4%	2.0
	B4	Ar + O_2	15	113%	171%	35.4%	1.9
	B6	Ar + CO_2	15	172%	292%	39.6%	3.1
10% H_2O_2	C1	Ar	5	155%	313%	47.1%	2.2
	C2	Ar	15	153%	307%	47.1%	2.7
15% H_2O_2	D1	Ar	5	144%	428%	69.4%	5.1
	D2	Ar	15	227%	500%	51.6%	5.1

3.3 Antibacterial properties

Bactericidal effect of modified MC samples prepared with different plasma-chemical procedures was compared with original MC cellulose. Luminometric measurements show a decrease of bacterial bioluminescence as *E. coli* viability marker in all experiments. Firstly, the effect of plasma-chemical reaction duration (5 or 15 min) of group samples A (in water) and B (5% H_2O_2) was evaluated (Fig. 4). It is clearly visible that hydrogen peroxide significantly decreases the *E. coli* viability in comparison with distilled water resulting in much higher antibacterial effect. Moreover, as it is visible from comparison of samples A1 and A2 (18.6 and 34.7% mortality of *E. coli*, respectively), the longer plasma-chemical treatment the more efficient is the bacteria killing. The presence of only 5% H_2O_2 already increased the bactericidal effect of MMC to approx. 95% (T-test, $p<0.01$).

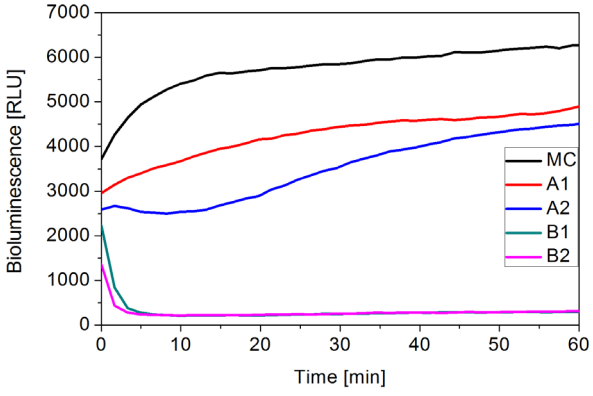

Figure 4: Kinetic curves representing the bioluminescence of *E. coli* within first hour of measurement in the presence of 5 mg MMC samples in water (A1, A2), and in 5% H_2O_2 (B1, B2) compared to original non treated cellulose (MC). Samples A1, B1 were treated for 5 min in plasma discharge and samples A2 and B2 for 15 min.

In the following experiment, the effect of admixture gas (none, O_2 or CO_2) on MC samples (5 mg) after 15 min. of plasma-chemical reaction in either distilled water (A sample series) or 5% H_2O_2 (B sample series) was compared (see Fig. 5). The results confirm the significant decrease of *E. coli* bioluminescence in the group A and B samples in comparison with the control sample, while the B group showed a greater effect than the samples of group A. However, there is not a significant difference among each sample within the group (A or B), proving that the admixture gas does not have any notable effect on the bioluminescence. Therefore, the samples without any admixture gas have been chosen for following experiments.

In the last set of experiments, the effect of different amounts of sample (5, 10 and 15 mg $well^{-1}$) after 15 min of plasma-chemical reaction in distilled water (samples A), 5% H_2O_2 (samples B), 10% H_2O_2 (samples C) and 15% H_2O_2 (samples D) on the MC bactericidal behaviours was tested.

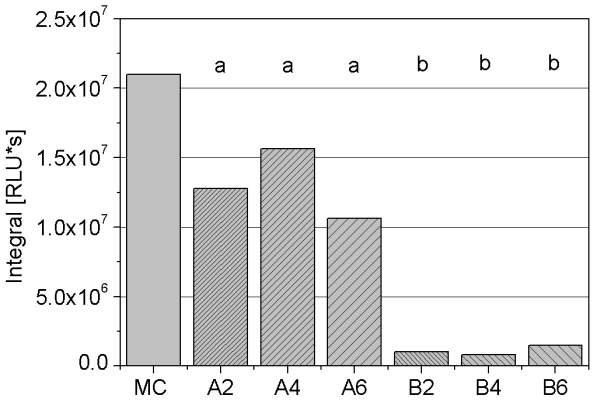

Figure 5: The effect of the admixture gas type on the MMC bactericidal activity. Different "a" and "b" letters show the significance in the decrease of *E. coli* bioluminescence between A (treated in water) and B (treated in 5% H_2O_2) samples towards the control (a: p<0.05, b: p<0.01) but not within A or B samples of the same group.

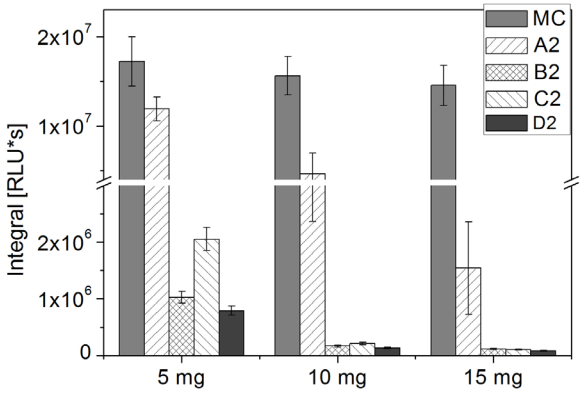

Figure 6: The effect of sample amount (5, 10 and 15 mg) and H_2O_2 concentration (MC – non-treated sample, A – pure water, B – 5%, C – 10%, D – 15% H_2O_2) on the MMC bactericidal activity. Results are presented as average integral ± S.D.

As shown in Fig. 6, direct proportion between the sample dose and antibacterial effect was observed in all tested samples, where the bactericidal activity was decreased by about 99% for samples with 10 and 15 mg $well^{-1}$ at the presence of hydrogen peroxide (groups B, C and D). Increasing the amount of H_2O_2 also resulted in higher *E. coli* mortality.

4 Conclusions

Based on the results above, it can be concluded that plasma-chemical treatment of microcrystalline cellulose led to a chemical modification obtaining samples with unique bactericidal effects. ATR characterisation discovered one extra absorption band for modified MC characteristic of the oxidation process in the cellulose material. Increased presence of carboxyl groups was also confirmed by the titration method. Generally, major effect of the oxidation process in the suspension of microcrystalline cellulose grown with increasing concentration of hydrogen peroxide. In conclusion, we can summarize that presence of admixture gas slightly supports the oxidation process (in the order Ar>Ar+O2>Ar+CO2) and with time of plasma treatment but do not have a significant impact on the final COOH content. Plasma treatment of microcrystalline cellulose in the pure water led to the mild oxidation process which resulted in increasing both the COOH integrated area and bactericidal effect. The bacterial activity in this case decreased about 25-50%. The presence of H_2O_2 in experiments caused a significant bactericidal effect, where the viability of *E. coli* in all samples decreased about 99%. In our basic dose-dependent test (Fig. 6) we found that a small dose (5 mg $well^{-1}$) of modified MC has visible antibacterial properties. Concentration of hydrogen peroxide in suspensions and time of plasma treatment do not have a significant influence on the resulting antibacterial properties. The presented new plasma-oxidizing method of MC without toxic solvents may have great potential in medicine and mainly in tissue regeneration.

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